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Pili of a *Vibrio parahaemolyticus* Strain as a Possible Colonization Factor

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Pili of *Vibrio parahaemolyticus* were purified from a Kanagawa phenomenon-positive strain (Ha7) that belongs to serogroup O2:K3 and is adhesive to rabbit intestine. The organisms treated with the Fab fraction of antipilus antibody failed to adhere to the intestine. Purified pili had the ability to adhere to the intestine, but the pretreatment of the intestine with purified pili did not allow adherence of the organisms to the intestine. These results suggest that pili of this *V. parahaemolyticus* strain play an important role in colonization.

Vibrio parahaemolyticus, an important enteropathogen that causes acute gastroenteritis, was first isolated by Fujino et al. in 1950 from a mass outbreak of food poisoning in which 20 of the 272 victims died (9). Disease caused by this organism is widely distributed throughout the world. The isolation frequency of *V. parahaemolyticus* from food poisoning in Japan has been higher than that of any other enteropathogen (18). The pathogenicity of the organism with regard to exotoxins such as thermostable direct hemolysin has been intensively studied, and the details of thermostable direct hemolysin as a pathogenic factor have been clarified (14, 24, 25, 29, 31). Recently, another type of hemolysin (thermostable direct hemolysin-related hemolysin) in Kanagawa phenomenon-negative strains has attracted the attention of some investigators (13, 26). However, the mechanism of colonization by this organism is poorly understood. Although there have been a few reports on the ability of *V. parahaemolyticus* to adhere to cultured cells (4, 15), neither a colonization factor nor a receptor has been mentioned so far. Many kinds of pili in various bacteria have been shown to be colonization factors (6–8, 12, 27, 30). Therefore, those who investigate the colonization factors of bacteria usually direct their attention to pili at first. We have found pili on the surfaces of *V. parahaemolyticus* Ha7 cells and purified them (22, 23). The purified pili were not hemagglutinative, but the organisms were adhesive to the intestine (22, 23). This paper describes the role of Ha7 pili in adhesion to the intestine.

MATERIALS AND METHODS

Bacterial strain. *V. parahaemolyticus* Ha7 isolated from a diarrheal patient was used. Strain Ha7 belongs to serogroup O2:K3 and is positive for the Kanagawa phenomenon.

Culture conditions. For production of pili, the organisms were precultured in stationary heart infusion broth (HIB; Eiken Co., Tokyo, Japan) at 25°C for 15 h (40 ml of HIB in a 100-ml Erlenmeyer flask). This preculture was inoculated into fresh HIB supplemented with 3% sodium chloride (400 ml of medium in a 3-liter Erlenmeyer flask) and was subsequently cultured at 37°C for 4 to 5 h with reciprocal shaking. The organisms for the adhesion test were directly cultured in HIB supplemented with 3% NaCl at 37°C for 15 h, although pilus production was slightly decreased.

Purification of pili. The pili were purified as previously reported (22); the purification procedure is the same as that for purification of *Vibrio cholerae* pili (16). Briefly, the crude pili collected from harvested cells by biomixer treatment and centrifugation were precipitated with acid (0.1 M acetate buffer, pH 4.0). The precipitate was treated with 5 M urea and then salted out by the addition of a small amount of ammonium sulfate (1 volume of saturated ammonium sulfate added to 9 volumes of pilus suspension).

Adhesion test. The ability of strain Ha7 to adhere to rabbit intestine was examined by the MASK method, as previously reported (21).

Adhesion inhibition test. The procedure of the adhesion inhibition test was the same as that of the adhesion test, except that organisms treated with antibody (Fab fraction) or intestine treated with purified pili was used. In one method, organisms (10^9/ml) suspended in KRT buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4 \cdot 7H_2O$, 2.7 mM $CaCl_2$, 10 mM Tris hydrochloride [pH 7.5]) (17) containing 2.5 mg of Fab fraction prepared from antipilus antibody per ml instead of intact antibody were used, because the antibody agglutinates the organisms. In another method, intestine pretreated in pilus suspension (0.5 mg/ml) was exposed to the intact organisms.

Electron microscopy. Scanning electron microscopy for counting adherent vibrios was described in a previous report (21). Transmission electron microscopy for the observation of pili was carried out as follows. Suspensions of whole cells or purified pili were negatively stained with 4% uranyl acetate on a carbon-coated Formvar grid; this was then allowed to dry in air. The preparations were examined with a JEM 2000EX electron microscope.

Immunohistological test. The ability of the purified pili to adhere to the intestinal epithelium was examined by using horseradish peroxidase-labeled antibody. Two pieces of the intestine (one treated with purified pili and the other not) were prepared for histological examination. Paraffin-embedded specimens were sliced to a thickness of 5 to 10 μm and placed on a slide glass. After the paraffin was removed with xylene, the specimen was exposed first to rabbit antipilus antibody, then to peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG), and finally to diaminobenzidine tetra-hydrochloride or hydrogen peroxide, depending on the routine technique.

Antibodies. Antipilus antibody was prepared by immunizing rabbits with purified pili. The antibody was highly specific for the pili, as confirmed by Western blotting (immunoblotting) (33) of whole-cell lysate. Horseradish peroxi-

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FIG. 1. Scanning electron micrograph of *V. parahaemolyticus* adhered to rabbit small intestine (MASK method [21]). Bar, 50 μm.

dase-conjugated anti-rabbit IgG and gold colloid-labeled anti-rabbit IgG [$F(ab')_2$] (Organon Teknika, Malvern, Pa.) were commercially purchased.

Protein determination. Protein content was assayed by the method of Lowry et al. (19) with bovine serum albumin as the standard.

RESULTS

Adhesion to the intestine. Many vibrios were adherent to the villus surface, with an adhesion index of more than 100 (Fig. 1). Under higher magnification, fasciculate piluslike structures radiating from the vibrio cells were seen (Fig. 2), whereas the pili of the organisms cultured in HIB looked like fine threads (Fig. 3). Piluslike structures connected vibrio cells and villus surfaces.

Purified pili. The molecular weight of the subunit protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be about 17,000 (Fig. 4A). The antipilus antibody examined by Western blotting revealed a single band reactive against the crude sample as well as one against the purified pili, suggesting that the antibody has high specificity (Fig. 4B). The purified pili, as seen on the living cells, looked long and flexible and had diameters of 7 nm (Fig. 4C).

Adhesion inhibition. Strain Ha7 adhered well to the intestine in the examination by the MASK method. Pretreatment of Ha7 with the Fab fraction of anti-Ha7 pilus antibody inhibited adhesion markedly. The inhibition was statistically significant, with a probability of less than 0.005. Fab fraction prepared from normal rabbit IgG did not inhibit adhesion. When the intestine was pretreated with purified pili, adhesion was also significantly inhibited (Table 1). Pili and Fab inhibited adhesion in a dose-dependent manner (Table 2).

Adhesion of purified pili. Whether purified pili adhered to the intestine was determined by using peroxidase-conjugated antibody. The intestine treated with purified pili developed a marked enzyme reaction on the villus surface, but untreated intestine did not (Fig. 5). The specimens were also examined by scanning electron microscopy, which revealed piluslike structures scattered on the epithelial surface of the intestine treated with pili; these structures were not seen in untreated intestine (Fig. 6).

Conjunction of Fab to pili. The conjunction of Fab and pili was confirmed by using gold colloid-labeled antibody. Treat-

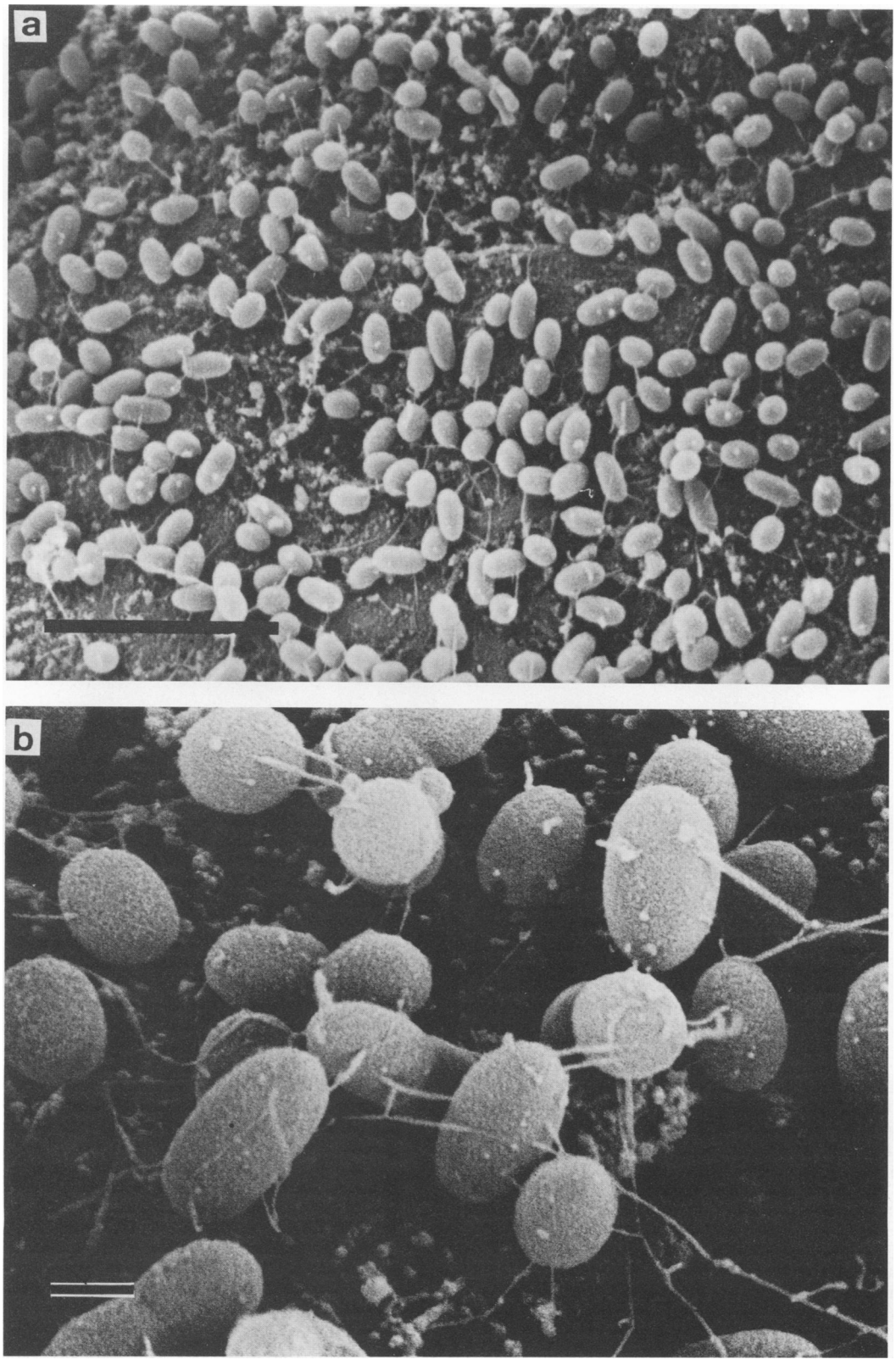

FIG. 2. Higher magnification of Fig. 1. Bars, 5 (a) and 0.5 (b) μm.

FIG. 3. Negative staining of *V. parahaemolyticus* Ha7 cultured in HIB. Bar, 0.5 μm.

ment of organisms with antipilus Fab made the pili fat, suggesting that they conjugated Fab. Also, the Fab on pili was visualized by gold colloid particles lining the pili (Fig. 7). No positive findings were obtained on Fab preparations from normal rabbit IgG.

DISCUSSION

Colonization of the intestine by enteropathogens is an initial step in the infectious process of acute gastroenteritis. However, colonization of the intestine by *V. parahaemolyticus* has never been reported, as far as we know. Several investigators have studied the property of *V. parahaemolyticus* adhesion to cultured tissue cells or chitin (3, 4, 10, 15, 20, 28), and they dicussed the possibilities that the adherence is mediated by lateral flagella, the outer membrane, or capsular antigen, without mentioning pili (3–5). Recently, we and Honda et al. found pili on the cell surface of *V. parahaemolyticus* (11, 22), and we purified them (22).

To determine whether pili have a role in colonization, we used organisms cultured in broth to avoid the influence of lateral flagella, which are occasionally produced on solid medium (1–3). We confirmed in the preliminary examinations that the separated flagella or outer membrane fraction of *V. parahaemolyticus* Ha7 was not adhesive to the intestine and did not inhibit the adherence of the organisms (data not shown).

This study demonstrated that Ha7 cultured in broth was adhesive to the intestine. The adhesion was inhibited by treating the organisms with antipilus antibody (Fab) or by blocking the epithelial receptor with purified pili. The inhibition was dependent on the dose of Fab or pili. CFA/I pili of

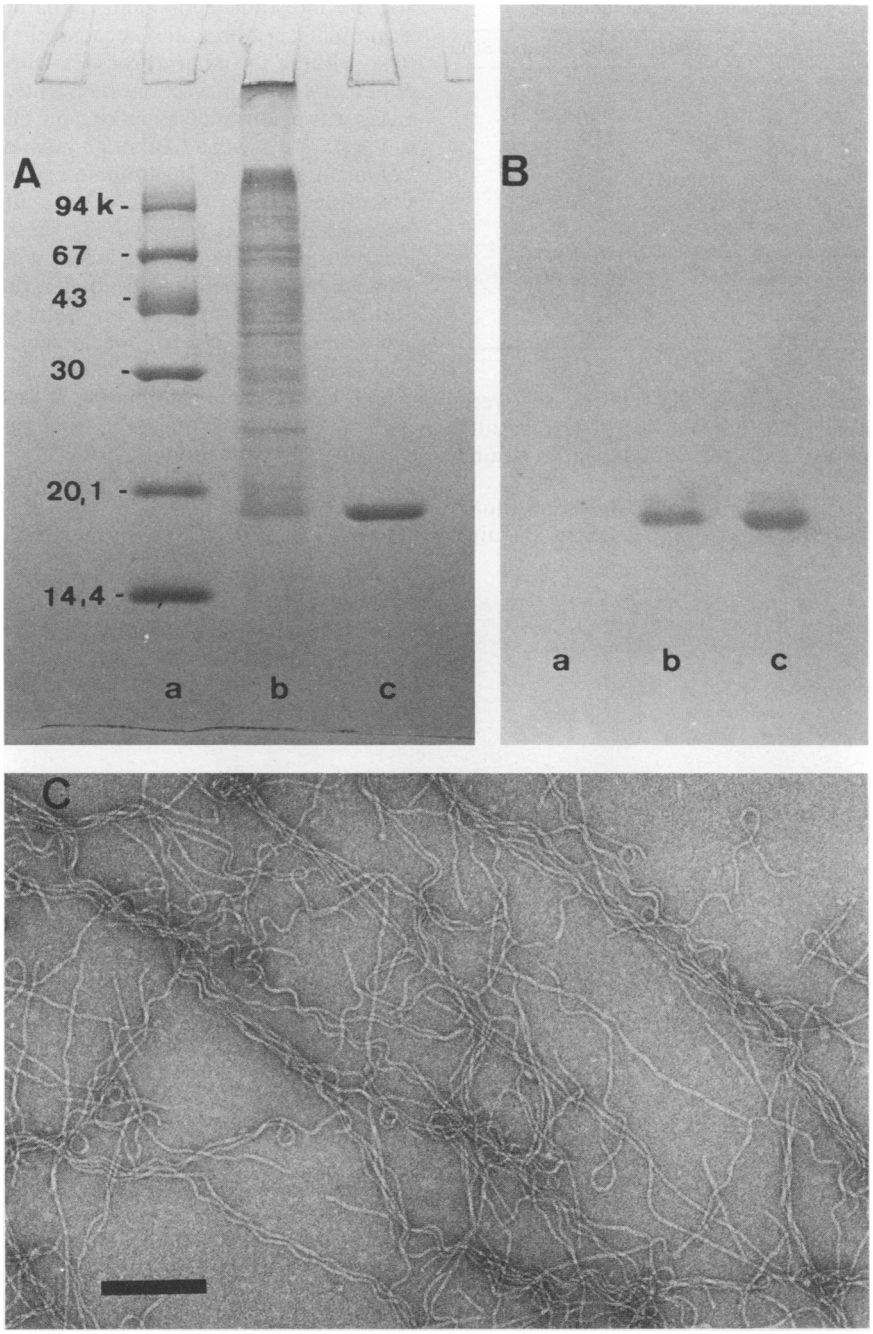

FIG. 4. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the subunit protein. Lane a, Molecular size marker; lane b, pellet obtained by acid precipitation; lane c, purified pili. k, Kilodaltons. (B) Western blot of the gel shown in panel A with antipilus antibody. (C) Electron micrograph of the purified pili. Bar, 200 nm.

enterotoxigenic *Escherichia coli* were shown to be a colonization factor by essentially the same method as that used in the present study (8). Therefore, it can be said that pili are most likely a colonization factor of *V. parahaemolyticus* Ha7.

To examine the distribution of Ha7-type pili, whole-cell lysates of 12 strains (5 from patients and 7 from seawater) were examined by Western blotting with anti-Ha7 pilus antibody. All strains except Ha7 gave a negative reaction;

TABLE 1. Adhesion inhibition tests with purified pili or Fab fraction

Expt no.	Adhesion index (mean ± SD) for:			
	Nontreated intestine	Pure pili	Normal-Fab fraction	Pili-Fab fraction
1	156 ± 45	10 ± 9	167 ± 44	23 ± 21
2	125 ± 94	20 ± 13	115 ± 34	41 ± 30
3	197 ± 45	35 ± 17	244 ± 25	37 ± 25

TABLE 2. Dose dependency of adhesion inhibition

Concn (μg/ml)	Adhesion index (mean ± SD)
Pili	
0	197 ± 45
125	227 ± 55
250	154 ± 42
500	35 ± 17
Fab	
0	156 ± 34
625	134 ± 21
1,250	66 ± 20
2,500	8 ± 5

nevertheless, those strains possessed flexible pili and were adhesive to the intestine (data not shown). These results suggest that other colonization factors are present in strains other than Ha7.

Strain Ha7 (whole cells and purified pili) did not agglutinate human or sheep erythrocytes despite its strong affinity for the intestinal epithelium. Separated CFA/I pili do not agglutinate erythrocytes because they are monovalent (8). They adhere to the erythrocytes without hemagglutination. Whether Ha7 pili were monovalently adhesive to erythrocytes was determined by mixing pili and erythrocytes, but no adsorption was seen. Although most adhesive pili and other colonization factors agglutinate erythrocytes (6–8, 27), colonization factors without hemagglutinating activity have occasionally been reported (12, 28, 32). Honda et al. reported a new colonization factor isolated from enterotoxigenic *E. coli* which had no hemagglutinating activity (12). Reyes et al. reported *V. parahaemolyticus* strains which were adhesive to human buccal cells but not hemagglutinative (28). Teppema et al. reported a *V. cholerae* O1 strain which has no hemagglutinating activity but is highly adhesive to rabbit intestine (32). Ha7 pili may belong to this category of colonization factor.

Honda et al. also reported partially purified pili from *V. parahaemolyticus* (11). Although they did not mention the adhesive property of pili, they reported that almost all

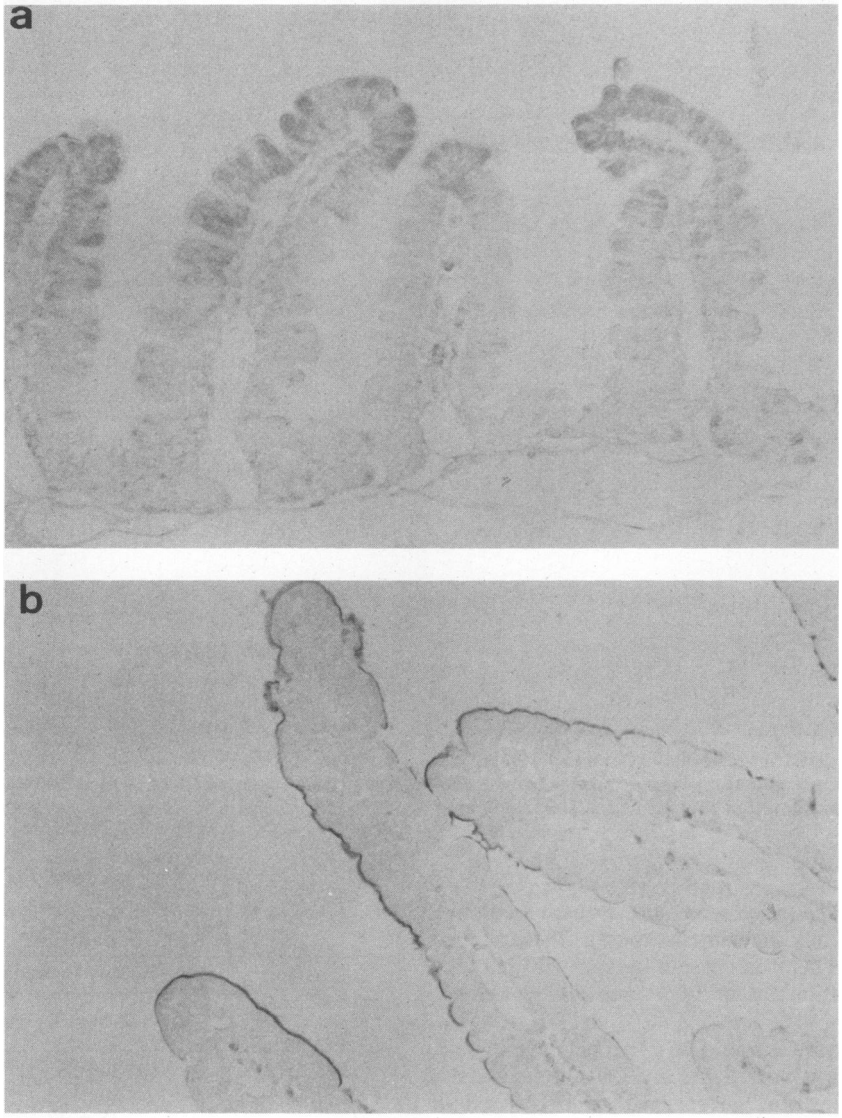

FIG. 5. Immunohistological findings. (a) Control (untreated) intestine; (b) intestine treated with purified pili.

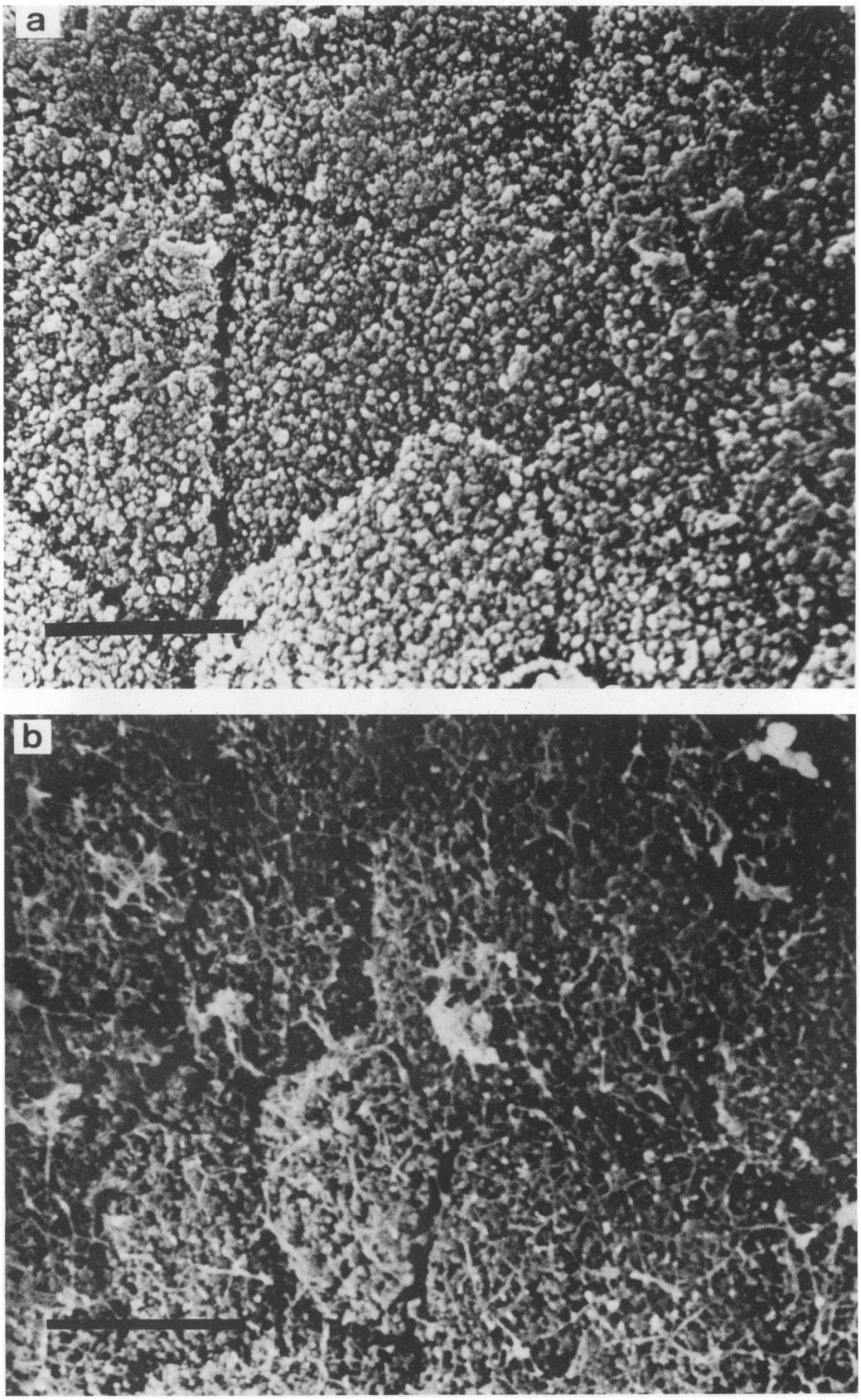

FIG. 6. Scanning electron micrograph of intestine. (a) Control (untreated) intestine; (b) intestine treated with purified pili. Bars, 0.5 μm.

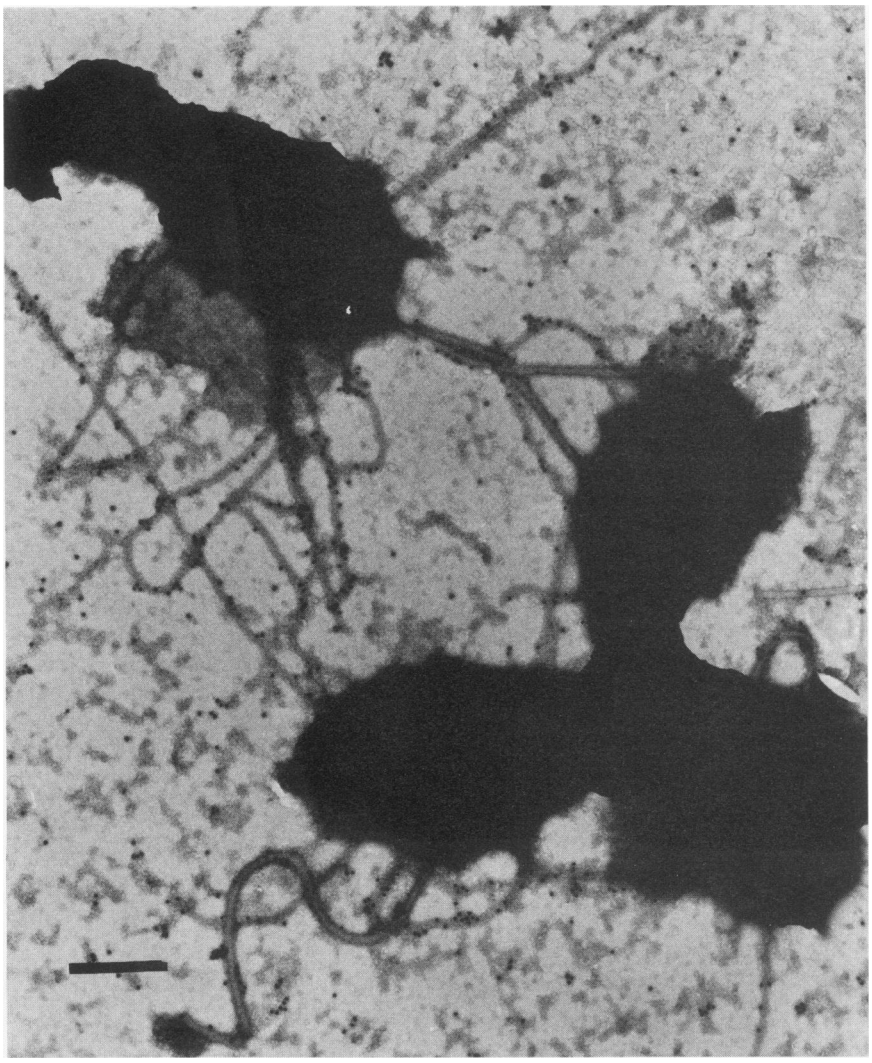

FIG. 7. Immunogold labeling for the detection of antipilus antibody Fab. Bar, 0.5 μm.

strains of *V. parahaemolyticus* isolated from either patients or the environment possessed pili. Characterization of pili of strains other than Ha7 compared with other colonization factors and a comparison with Ha7 pili are now required.

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